Esculentin-2CHa(1–30) and its analogues: stability and mechanisms of insulinotropic action

Vasu, S., McGahon, M. K., Moffett, R. C., Curtis, T. M., Conlon, J. M., Abdel-Wahab, Y. H. A., & Flatt, P. R. (2017). Esculentin-2CHa(1–30) and its analogues: stability and mechanisms of insulinotropic action. *The Journal of endocrinology*, *232*, 423-435. https://doi.org/10.1530/JOE-16-0453

Published in:
The Journal of endocrinology

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

Publisher rights
© 2017 Society for Endocrinology.
Disclaimer: this is not the definitive version of record of this article.This manuscript has been accepted for publication in Journal of Endocrinology, but the version presented here has not yet been copy-edited, formatted or proofed. Consequently, Bioscientifica accepts no responsibility for any errors or omissions it may contain. The definitive version is now freely available at doi.org/10.1530/JOE-16-0453 2017

General rights
Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Download date:26. Feb. 2021

Esculentin-2CHa(1-30) and its analogues – stability and mechanisms of insulinotropic action

Srividya Vasu[1*], Mary K. McGahon[2], R. Charlotte Moffett[1], Tim M. Curtis[2], J. Michael Conlon[1], Yasser H. A. Abdel-Wahab[1] and Peter R. Flatt[1]

[1]SAAD Centre for Pharmacy & Diabetes, School of Biomedical Sciences, University of Ulster, Coleraine, BT52 1SA, UK

[2]Centre for Experimental Medicine, Queens University of Belfast, Belfast, BT9 7BL, UK

***Corresponding author**:

E-mail: s.vasu@outlook.com

Short title: Esculentin-2CHa(1-30) & its analogues

Keywords: Esculentin, insulin secretion, glucose tolerance, diabetes, amphibian peptide, pancreatic beta cells

Word count: 4777

Abstract

The insulin-releasing effects, cellular mechanisms of action and anti-hyperglycaemic activity of 10 analogues of esculentin-2CHa lacking the cyclic C-terminal domain (CKISKQC) were evaluated. Analogues of the truncated peptide, esculentin-2CHa(1-30), were designed for plasma enzyme resistance and increased biological activity. Effects on insulin release, cell membrane integrity, membrane potential, intracellular Ca^{2+} and cAMP levels were determined using clonal BRIN-BD11 cells. Acute effects on glucose tolerance were investigated using NIH Swiss mice. D-amino acid substitutions at positions 7(Arg), 15(Lys) and 23(Lys) and fatty acid (L-octanoate) attachment to Lys at position 15 of esculentin-2CHa(1-30) conveyed resistance to plasma enzyme degradation whilst preserving insulin-releasing activity. Analogues [D-Arg^{7}, D-Lys^{15}, D-Lys^{23}]-esculentin-2CHa(1-30) and Lys^{15}-octanoate-esculentin-2CHa(1-30) exhibiting most promising profiles and with confirmed effects on both human insulin-secreting cells and primary mouse islets were selected for further analysis. Using chemical inhibition of adenylate cyclase, protein kinase C or phospholipase C pathways, involvement of PLC/PKC mediated insulin secretion was confirmed similar to that of CCK-8. Diazoxide, verapamil and Ca^{2+} omission inhibited insulin secretion induced by the esculentin-2CHa(1-30) analogues suggesting an action also on K_{ATP} and Ca^{2+} channels. Consistent with this, the analogues depolarised the plasma membrane and increased intracellular Ca^{2+}. Evaluation with fluorescently labelled esculentin-2CHa(1-30) indicated membrane action, with internalisation, but patch clamp experiments suggested that depolarisation was not due to direct inhibition of K_{ATP} channels. Acute administration of either analogue to NIH Swiss mice improved glucose tolerance and enhanced insulin release similar to that observed with GLP-1. These data suggest that multi-acting analogues of esculentin-2CHa(1-30) may prove useful for glycaemic control in obesity-diabetes.

Introduction

Incidence of type 2 diabetes is constantly on the rise, owing to an increase in consumption of a western diet, sedentary lifestyle, obesity and aging population (Stumvoll *et al.* 2008, McCarthy, 2010). Current therapies targeting beta-cell secretory function and/or insulin action offer metabolic benefits but due to inability to restore normal glycaemic control, diabetes associated complications arise including cardiovascular disease, neuropathy, nephropathy and retinopathy **(**McCarthy, 2010, Parkes *et al.* 2013, Kahn *et al.* 2014**)**. As a result, there is a constant need for development of new, improved therapeutic agents to complement or replace existing anti-diabetic drugs. Peptide hormone therapeutics and various glucagon-like peptide-1 (GLP-1 mimetics), have been strongly promoted over the past few years (Kahn *et al.* 2014, Irwin & Flatt, 2015). This approach has several potential advantages over development of small molecule drugs, providing greater specificity and improved safety (Parkes *et al.* 2013).

In the 1980s, the search for bioactive agents in venoms of insects and reptiles led to the isolation and characterisation of exendin-4 from the salivary secretions of *Heloderma suspectum* (Gila monster) (Conlon *et al.* 2006). This peptide has been shown to stimulate insulin secretion and exert a range of glucoregulatory actions in a fashion similar to incretin hormone, GLP-1 (Parkes *et al.* 2013). Subsequently, long acting GLP-1 mimetics with good clinical efficacy and acceptable benefit-risk profiles have been developed for treatment of patients with type 2 diabetes (Irwin & Flatt, 2015). The search for naturally occurring bioactive agents has continued to date. Skin secretions of frogs and toads are a potentially valuable source of peptides that hold great therapeutic potential. Such molecules synthesized in the skin of amphibians (particularly the Hylidae (Nicolas & El Amri, 2009, Jackway *et al.* 2011), Pipidae (Mechkarska *et al.* 2010), and Ranidae (Conlon, 2008, Conlon, 2011) families) are well known for their antimicrobial, antiviral, anti-tumor, immunomodulatory and chemoattractive properties (Conlon *et al.* 2014). In addition, we have demonstrated that some of these host defence peptides isolated from frog skin secretions were insulinotropic *in vitro* and could improve glucose tolerance in animal models *in vivo* (Conlon *et al.* 2014).

Esculentin-2CHa (GFSSIFRGVAKFASKGLGKDLAKLGVDLVACKISKQC), isolated from norepinephrine-stimulated skin secretions of the Chiricahua leopard frog, *Lithobates chiricahuensis* (Ranidae), has been shown to exhibit potent antimicrobial activity against clinical isolates of multidrug-resistant strains of *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia* (Conlon *et al.* 2011). In addition, this bioactive peptide also stimulated interleukin-10 (IL-10) release by mouse lymphoid cells and exerted cytotoxicity against human non-small lung adenocarcinoma A549 cells with low haemolytic activity against human erythrocytes (Attoub *et al.* 2013). Increasing the cationicity of the peptide with L-Lysine substitution of Asp^{20} and Asp^{27} residues enhanced antimicrobial activity while removal of either the hydrophobic N-terminal hexapeptide (GFSSIF) or the cyclic C-terminal domain (CKISKQC) and serine substitution of Cys^{31} and Cys^{37} residues decreased antimicrobial potency (Attoub *et al.* 2013).

We recently reported anti-diabetic effects of an analogue of esculentin-2CHa – [Lys28]-esculentin-2CHa in high fat fed diabetic mice (Ojo *et al.* 2015c). Our previous observations indicate that any modification of frog skin peptides resulting in loss or reduction of antimicrobial activity also resulted in compromise of insulinotropic action. Interestingly, our preliminary observations revealed that loss of antimicrobial activity associated with removal of the cyclic C-terminal domain of esculentin-2CHa was not accompanied by abolition of insulinotropic actions *in vitro*. In other words, the truncated form of esculentin-2CHa with 30 amino acid residues (esculentin-2CHa-GA30) and lacking the C-terminal disulphide bond stimulated insulin release from BRIN-BD11 cells.

Based on this and with a view to generating more easily synthesised/cost effective forms of esculentin-2CHa with potential as a possible new class of therapeutic peptides for diabetes, we designed a family of 10 analogues of esculentin-2CHa(1-30) as indicated in Table 1. D-isomers of naturally occurring amino acids were substituted at positions 7, 15 and 23 (Peptides 2-6) to confer resistance to endopeptidases based on the observed degradation pattern of the peptide in plasma. In addition, lysine residues at positions 15 and 23 were substituted with L-ornithine with a view to increasing metabolic stability (Peptide 7) and amidation of C-terminus (Peptide 8). To prolong half-life in the circulation (by facilitating binding to serum albumin), analogues were synthesised with a C-8 fatty acid (octanoate) attached to the lysine residue at position 15 or 23 (Peptides 9 or 10. Using the parent esculentin-2CHa(1-30) (Peptide 1) as positive control, we investigated these various modified analogues for enzymatic stability, insulinotropic effects, cellular mechanisms of action and acute antihyperglycaemic effects *in vivo*.

Materials and methods

Peptide synthesis and purification: Synthetic esculentin-2CHa(1-30) and analogues (Table 1) were purchased (> 95 % pure) from GL Biochem Ltd (Shanghai, China) and purified to near homogeneity (> 98 % pure) by reversed-phase HLPC on a (2.2 cm x 25 cm) Vydac 218TP1022 (C18) column equilibrated with acetonitrile/water/triflouroacetic acid (TFA) (21.0/78.9/0.1 v/v) mobile phase at a flow rate of 1 ml/min. The concentration of acetonitrile in the eluting buffer was raised to 56% (v/v) over 60 min. The molecular masses of the peptides were confirmed using MALDI-TOF mass spectrometry (Table 1). Other peptides including the enzyme resistant form of CCK-8, pggCCK-8 (Irwin *et al.* 2013) were purchased from American Peptide Company (Sunnyvale, CA, USA).

Peptide degradation studies: Susceptibility of esculentin-2CHa(1-30) and related peptides to plasma proteolytic enzymes was determined by incubating the peptides with plasma (10 µl) from fasted NIH Swiss mice in 50 mM triethanolamine-HCl buffer (pH 7.8) at 37 °C (O'Harte *et al.* 2001) for 0/8 h. The reactions were stopped by adding 10% (v/v) TFA/water (10 µl). Separation of intact and degraded products was carried out using reversed phase HPLC with a Vydac C-18 column equilibrated with 0.12% (v/v) TFA/water at a flow rate of 1.0 ml/min. The concentration of acetonitrile in the eluting solution was increased over a linear gradient from 0 to 28% in 10 min, to 56% in 20 min and from 56% to 70% in 5 min. MALDI-TOF mass spectrometry was used to ascertain the molecular masses of both intact and degraded products.

Cell culture: Insulin-secreting BRIN-BD11 rat clonal beta cells and 1.1B4 human clonal beta cells were routinely cultured in RPMI-1640 medium supplemented with 10 % (v/v) FBS and 1 % (v/v) antibiotics – penicillin (100 U/ml) and streptomycin (0.1 mg/ml). The generation, culture and characteristics of these two cell lines have been described previously (McClenaghan *et al.* 1996, McCluskey *et al.* 2011)

***In vitro insulin-releasing studies*:** *In vitro* insulin-releasing effects of esculentin-2CHa(1-30) and its analogues were assessed using clonal beta cell lines as well as isolated mouse pancreatic islets. Firstly, BRIN-BD11 cells were incubated with the peptides in the concentration range ($1 \times 10^{-12} – 3 \times 10^{-6}$M) in Krebs-Ringer bicarbonate buffer (KRBB) containing 5.6mM glucose for 20 min at 37 °C as previously described (Abdel-Wahab *et al.* 2008, Mechkarska *et al.* 2011, Ojo *et al.* 2011). Effects of established modulators of insulin release, removal of extracellular Ca^{2+} and inhibitors of phospholipase C (U73122) and adenylate cyclase (NKY80) were also tested (Abdel-Wahab *et al.* 2008, Mechkarska *et al.* 2011, Ojo *et al.* 2011). Plasma membrane integrity was assessed by measuring lactate dehydrogenase (LDH) in cell incubation buffer using CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. In a second set of experiments, insulin releasing effects of esculentin-2CHa(1-

30) and selected analogues were examined over a similar concentration range using 1.1B4 human clonal beta cells (McCluskey *et al.* 2011, Green *et al.* 2015). In a third set of experiments, pancreatic islets isolated from NIH Swiss mice by collagenase digestion (Gotoh *et al.* 1985), were incubated with 10^{-6} and 10^{-8}M of esculentin-2CHa(1-30) and selected analogues for 1 h in Krebs-Ringer bicarbonate (KRB) buffer supplemented with 3 or 20 mM glucose. Other experiments detailed below were conducted at peptide concentration of 10^{-6}M which elicited prominent insulin secretory effects. Insulin release was measured by radioimmunoassay (Flatt & Bailey, 1981a, Flatt & Bailey, 1981b) using mouse or human insulin standards as appropriate.

Membrane potential studies and intracellular calcium ($[Ca^{2+}]_i$): Effects of esculentin-2CHa(1-30) and analogues on membrane potential and intracellular calcium $[Ca^{2+}]_i$ were assessed using BRIN-BD11 cells (FLIPR membrane or calcium assay kit, Molecular Devices, USA) as previously described (Miguel *et al.* 2004). BRIN-BD11 cells were incubated with Krebs-Ringer bicarbonate buffer containing 5.6mM glucose. Esculentin-2CHa(1-30) and its analogues were added, with calcium mobilisation data collected and analysed using Softmax Pro software (Miguel *et al.* 2004).

Membrane binding and patch-clamp electrophysiology

For membrane binding studies, BRIN–BD11 cells were seeded onto polysine coated slides (40,000 cells/slide) and cultured overnight. Media was replaced with KRBB containing 1 μM FITC-esculentin-2CHa(1-30) and incubated for 5-90 minutes. Coverslips were washed with PBS, rapidly transferred to the recording bath (containing fresh PBS) mounted on an inverted microscope (Leica DMI6500B) coupled to a Leica TCS SP5 II confocal. Cells were excited by an argon laser (488nm) and simultaneously viewed on the transmitted light channel to allow assessment of the distribution of FITC-esculentin-2CHa(1-30) on plasma membrane and cytosolic compartments of the cells. Ionic currents were recorded from BRIN-BD11 pancreatic β-cells using the whole-cell mode of the patch clamp technique as previously described (Ojo *et al.* 2016). Amphotericin B was included in the pipette solutions to perforate the membrane and reduce current run-down such that currents were stable for the duration of the recording (Ojo *et al.* 2016). Current densities were calculated by dividing current amplitudes by the whole-cell capacitance (6-19 pF). External drug containing solutions were applied using a gravity-driven perfusion system with an exchange time of approximately 1s (Scholfield & Curtis, 2000). K_{ATP} currents were elicited by ramp protocols from +20 to -80 mV applied over 1 second from a holding potential of 0 mV using high K^+ external solution (containing in mM: 130 KCl, 10 TEACl, 2.5 Glucose, 1.3 $MgCl_2$, 2 $CaCl_2$, 10 HEPES pH 7.4 with NaOH). 100nM penitrem A, 1mM 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) and 1μM nimodipine were added to inhibit BK, Cl^- and L-Type Ca^{2+} channels and a K^+-based internal (pipette) solution was used (130 KCL, 1 $MgCl_2$, 0.045 $CaCl_2$, 1 EGTA, 10 HEPES, pH 7.2 with NaOH). K_{ATP} channel opening was stimulated with 200μM diazoxide prior to, and during application of 1μM [D-Arg^7, D-Lys^{15}, D-Lys^{23}]-esculentin-2CHa(1-30) (Peptide 6).

In vivo studies

Adult male National Institutes of Health (NIH) Swiss mice (Harlan Ltd, UK) were housed individually in an air-conditioned room (22 ± 2 °C) with a 12-hour light: 12-hour dark cycle and maintained on a standard rodent diet (Trouw Nutrition, Cheshire, UK), with food and water available *ad libitum*. For acute *in vivo* studies, overnight fasted mice received an intraperitoneal injection of glucose alone (18 mmol/kg body weight) or in combination with esculentin-2CHa(1-30) or its analogues (75 nmol/kg body weight). This dose was chosen on the basis of results in previous studies examining glucoregulatory effects of amphibian skin peptides (Conlon *et al.* 2014). A small dose-response study was conducted using GLP-1 and the two most prominent glucose-lowering peptides (Peptides 6 and 9). Blood samples were collected before injection and at times indicated in the Figures. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and 'Principles of laboratory animal care' (NIH publication no. 86 – 23, revised 1985).

Statistical analysis: Results were analysed using GraphPad PRISM Software (Version 6.0) and presented as mean ± S.E.M. Statistical analyses were performed using student's t test (non-parametric) or one-way ANOVA followed by Bonferroni or Student-Newman-Keuls post hoc test wherever applicable. Area under the curve (AUC) analysis was performed using the trapezoidal rule with baseline correction. Membrane current-voltage relations were compared using 2-way repeated measures ANOVA with Bonferroni *post hoc* test. Results were considered significant if $p < 0.05$.

Results

Plasma stability of esculentin-2CHa(1-30) and analogues:

Degradation of esculentin-2CHa(1-30) (Peptide 1) exposed to mouse plasma was 93% in 8 hours (Table 2). Examination of degradation products by mass spectrometry suggests that the native peptide is cleaved by enzymes at the following sites: between Phe^{6} and Arg^{7}, $\mathrm{Arg}^{7}$and Gly^{8}, Lys^{11} and Phe^{12}, Ser^{14} and Lys^{15}, Leu^{17} and Gly^{18}, Ala^{22} and Lys^{23} and Leu^{28} and Val^{29}. Substitution with D-isomers of residues at position 7 (Peptide 2), position 15 (Peptide 3) and positions 7, 15 and 23 (Peptide 6) conferred resistance to degradation, with degradation ranging between 24-59% (Table 2). Substitution with D-lysine residues at position 23 (Peptide 4) and at positions 15 and 23 (Peptide 5) reduced degradation to approximately 80% (Table 2). Peptide 6 was cleaved only at Lys^{11} and Phe^{12} and Leu^{28} and Val^{29} compared to esculentin-2Cha-GA30, thus substitution of residues with D-isomers at these positions protected the sites from enzymatic cleavage. Substitution of lysine residues at positions 15 and 23 with L-ornithine (Peptide 7) and amidation of C-terminus (Peptide 8) did not confer resistance to degradation (Table 2). Addition of a C-8 fatty acid to lysine residue at position

15 (Peptide 9) or 23 (Peptide 10) conferred resistance to degradation (62 and 79% respectively, Table 2), with cleavage only at sites between $Arg^7$and Gly^8, Ala^{22} and Lys^{23} and Leu^{24} and Gly^{25} and $Arg^7$and Gly^8 and Leu^{24} and Gly^{25} respectively.

Insulinotropic actions of esculentin-2CHa(1-30) and analogues:

Esculentin-2CHa(1-30) (Peptide 1) and analogues stimulated insulin release from BRIN-BD11 cells significantly compared to respective control at glucose (5.6 mM) ($p<0.05$, $p<0.01$, $p<0.001$, Table 2). Substitution of residues at position 7 (Peptide 2), position 15 (Peptide 3), position 23 (Peptide 4) and positions 7 and 15 (Peptide 5) with respective D-isomers significantly increased insulin release from BRIN-BD11 cells ($p<0.01$, $p<0.001$, Table 2). Substitution with D-isomers at positions 7, 15 and 23 (Peptide 6) or with lysine residues at positions 15 and 23 with L-ornithine (Peptide 7) significantly increased insulin release from BRIN-BD11 cells compared with esculentin-2CHa(1-30) (Peptide 1) ($p<0.001$, Table 2). Amidation of C-terminus (Peptide 8) did not markedly affect insulin output from BRIN-BD11 cells compared to parent peptide (Table 2). Addition of a C-8 fatty acid to lysine residue at position 15 (Peptide 9) or 23 (Peptide 10) markedly increased insulin release from BRIN-BD11 cells ($p<0.001$, Table 2), with effects of Peptide 9 significantly greater than esculentin-2CHa(1-30) ($p<0.01$, Table 2). For native and all peptide analogues of esculentin-2CHa(1-30), threshold concentration for stimulating insulin release ranged between 10^{-7} M and $3x10^{-6}$ M (Table 2). Insulinotropic actions of esculentin-2CHa(1-30) and its analogues were comparable to that of GLP-1 (Table 2).

We confirmed that the insulinotropic actions of esculentin-2CHa(1-30) peptides were not due to cytotoxicity. Thus LDH release from BRIN-BD11 cells upon exposure to the peptides was similar to that observed in control incubations (Table 2). The only exception was Peptide 2 which appeared to induce significantly greater LDH release at $3x10^{-6}$ M

($p<0.001$, Table 2). From the in vitro stability and insulin release studies, substitution of residues at positions 7, 15 and 23 (Peptide 6) with respective D-isomers and addition of a C-8 fatty acid to lysine residue at position 23 (Peptide 9) appeared to confer greater plasma stability and insulinotropic action on esculentin-2CHa-GA30. As a result, the native form and these two superior analogues were carried forward for further studies.

As shown in Figure 1A, esculentin-2CHa(1-30) and its analogues (Peptide 6, Peptide 9) markedly increased glucose stimulated insulin secretion from isolated mouse islets at 10^{-6} M concentration ($p<0.05$, $p<0.01$, Figure 1A). The effects induced were similar to those observed with stable forms of GLP-1 and CCK-8, namely exendin-4 and pggCCK-8 respectively ($p<0.01$, Figure 1A). The insulinotropic actions were clearly glucose dependent in the case of esculentin-2CHa(1-30) peptides which did not affect insulin secretion at 3 mM glucose even at high concentrations (Figure 1A). Esculentin-2CHa(1-30) (Peptide 1) and its analogues (Peptide 6, Peptide 9) also stimulated insulin release from human clonal beta cell line, 1.1B4 ($p<0.05$, $p<0.01$, $p<0.001$, Figure 1B). Threshold concentration for stimulation of insulin secretion from 1.1B4 cells for esculentin-2CHa(1-30) was 10^{-8} M whereas threshold concentrations for modified peptides were 10^{-11} M (Figure 1B). The maximal effect appeared less than that induced by 10^{-6} M exendin-4 from 1.1B4 cells (Figure 1B).

Mechanisms underlying insulinotropic actions of esculentin-2CHa(1-30) and analogues:

Effects on intracellular cAMP levels: GLP-1 and forskolin markedly increased intracellular cAMP levels in BRIN-BD11 cells ($p<0.001$, Figure 2A). In contrast, esculentin-2CHa(1-30) and its analogues (Peptide 6 and 9) did not have any appreciable effect on cAMP levels (Figure 2A).

Effects of drugs and ionic manipulation on insulinotropic activity **:**Forskolin, PMA, GLP-1, pggCCK, Peptide 1, Peptide 6 and Peptide 9 significantly increased insulin release from

BRIN -BD11 cells ($p<0.05$, $p<0.01$, $p<0.001$, Figure 2B). Overnight 18 h culture with PMA (10 nM) to down-regulate PKC pathways (McClenaghan *et al.* 2006) reduced PMA, pggCCK8, Peptide 1, Peptide 6 and Peptide 9 stimulated insulin secretion compared to routine culture ($p<0.05$, $p<0.01$, Figure 2B), In contrast, the insulin-releasing action of forskolin or GLP-1 was not attenuated. Consistent with this, the AC inhibitor, NKY80 only significantly inhibited GLP-1 induced insulin secretion ($p<0.05$, Figure 2C), whereas the PLC inhibitor, U73122X significantly reduced pggCCK8, Peptide 1, Peptide 6 and Peptide 9 induced insulin secretion ($p<0.05$, $p<0.01$, Figure 3A). The insulinotropic effect of GLP-1 was not impaired by U73122X. Since esculentin-2CHa(1-30) peptides still evoked small increase of insulin release in presence of NKY80, ionic pathways involved in insulin secretion were investigated.

Verapamil and diazoxide did not affect basal insulin secretion while IBMX, KCl and tolbutamide markedly increased insulin release from BRIN-BD11 cells ($p<0.05$, $p<0.01$, Figure 3A). Verapamil reduced pggCCK8, Peptide ~~2~~ 1 and Peptide ~~10~~ 9 induced insulin secretion ($p<0.05$, Figure 3A) while diazoxide reduced the insulinotropic effects of GLP-1, pggCCK8, Peptide 1 and Peptide 9 compared to control ($p<0.05$, $p<0.01$, $p<0.001$, Figure 3A). Peptide 6 potentiated IBMX-induced insulin secretion ($p<0.05$, Figure 3A) while none of the peptides altered the stimulatory insulin secretory responses from cells depolarised with 30 mM KCl (Figure 3A). GLP-1 and all peptides tested potentiated insulin secretion in the presence of tolbutamide ($p<0.05$, Figure 3A). Insulinotropic actions of GLP-1, pggCCK8 and all esculentin-2CHa(1-30) peptides were abolished in the absence of extracellular Ca^{2+} (Figure 3B).

Effects on membrane potential and intracellular Ca^{2+}: Esculentin-2CHa(1-30) and its analogues (Peptide 6 and 9) increased membrane potential and depolarised BRIN-BD11 cells compared to 5.6 mM glucose control ($p<0.05$, $p<0.01$, $p<0.001$, Figure 4A,B). This was accompanied by a significant increase in intracellular $[Ca^{2+}]_i$ ($p<0.05$, $p<0.001$, Figure 4C,D). The magnitude of the effects was markedly less than that induced by a depolarising concentration of KCl but similar to GLP-1 (Figure 4).

Actions at plasma membrane:

FITC-esculentin-2CHa(1-30) was used to monitor interactions of the peptide at plasma membrane sites on BRIN-BD11 cells. Representative images showing cells incubated for 5-90 min with the fluorescent tagged peptide are shown in Figure 5. Membrane binding by FITC-esculentin-2CHa(1-30) was evident on the membrane of discrete populations of cells after 5 min exposure, while fluorescence in cytoplasm of cells was also evident after 20mins incubation becoming progressive more intense over time up to 90mins, suggesting initial binding with the membrane followed by internalisation of the peptide. To probe further the membrane effects underlying changes in membrane potential and intracellular Ca^{2+}, we examined the actions of [D-Arg^{7}, D-Lys^{15}, D-Lys^{23}]-esculentin-2CHa(1-30) (Peptide 6) on BRIN-BD11 cells using patch clamp technique. This revealed that the depolarisation observed in Figure 4A was unlikely to be due to direct action of the peptide on K_{ATP} channels as when membrane current was recorded under selective recording conditions using the patch clamp technique, Peptide 6 (1 μM) had no effect on the amplitude of diazoxide activated K_{ATP} current measured at -80mV (Figure 6A) or mean current density at voltages between 20 and -80mV ($P>0.05$, Figure 6B,C).

Acute anti-hyperglycaemic activity of esculentin-2CHa(1-30) and analogues:

As shown in Figure 7A, B, Peptide 6 and Peptide 9 significantly reduced the glycaemic excursion ($p<0.05$) when administered together with glucose to overnight fasted NIH Swiss TO mice. This was associated with elevated insulin concentrations, with Peptide 9 significantly increasing integrated (AUC) plasma insulin values ($p<0.01$, Figure 7C,D). The effects observed were broadly similar to those induced by an equal dose of GLP-1 (Figure 7A-D). Follow-up dose-response studies revealed that 75 nmol/kg body weight was the minimal effective anti-hyperglycaemic dose of GLP-1, Peptide 6 or Peptide 9 under the experimental conditions employed ($p<0.05$, Figure 7E).

Discussion:

Genetic influences and lifestyle factors promote the constantly increasing incidence of type 2 diabetes, which is treated clinically by strategies that target pancreatic beta cell dysfunction and/or insulin resistance (Bailey, 2009, Irwin & Flatt, 2015). Recently peptide therapeutics for diabetes using stable mimetics of GLP-1 have received much attention due to their tolerability, potency and efficacy compared to small molecules drugs. Our recent observations reveal that esculentin-2CHa possesses potent insulinotropic actions and an analogue - [Lys28]-esculentin-2CHa, exerted beneficial effects on metabolism in high fat fed mice with insulin resistance and impaired glucose tolerance (Ojo *et al.* 2015c). We have observed that esculentin-2CHa(1-30), a truncated and more readily synthesised analogue of 30 amino acids lacking the cyclic C-terminal domain, retains insulin-releasing activity. The present study investigates the stability, insulinotropic actions and mechanisms of insulin secretion of esculentin-2CHa(1-30) and designer analogues together with their possible development for treatment of type 2 diabetes.

In vitro plasma degradation studies revealed that substitution with D-isomers of residues at position 7 (Peptide 2), position 15 (Peptide 3) and positions 7, 15, 23 (Peptide 6) and addition of a C-8 fatty acid to lysine residue at position 15 (Peptide 9) or position 23 (Peptide 10) enhanced resistance to degradation by plasma proteolytic enzymes. Peptides 6, 9 and 10 were partially degraded to 3 fragments after 8 h incubation with mouse plasma whereas esculentin-2CHa(1-30) was degraded to 5 fragments. Enhanced resistance to degradation coupled with intact insulinotropic activity may be beneficial *in vivo*. Indeed, insulinotropic actions of modified analogues were well preserved in clonal BRIN-BD11 cells. These actions were not associated with cellular cytotoxicity as indicated by lack of leakage of the intracellular marker LDH.

On the basis of enzymatic stability and insulin-releasing potency, three peptides were chosen for further evaluation, namely the analogue with triple D-isomer substitution (Peptide 6), the acylated form of esculentin-2CHa(1-30) (Peptide 9) and for comparison the parent molecule, esculentin-2CHa(1-30) (Peptide 1). Studies using isolated mouse islets highlighted the glucose-dependent insulin-releasing properties of all three peptides, which exerted effects similar to those of stable analogues of GLP-1 and CCK-8 (exendin-4 and pggCCK-8, respectively). When tested using the novel electrofusion-derived human 1.1B4 cell line (McCluskey *et al.* 2011), the esculentin-2CHa(1-30) peptides stimulated concentration-dependent insulin secretion with lower threshold stimulatory concentrations being observed for the modified analogues. These data indicate that these peptides should not induce hypoglycaemia are that they are likely to stimulate insulin secretion from human beta cells, with translational effects in vivo.

Beta cell stimulus-secretion coupling is a complex process, with the involvement of many key players including K_{ATP} channels, ATP, PKA, PKC, cAMP, Ca^{2+}, functional microtubule and microfilament system (McClenaghan, 2007, Fu *et al.* 2013). Beta cells detect changes in blood glucose levels and subsequent metabolism leads to increase in ATP levels that induces closure of plasma membrane K_{ATP} channels and depolarisation resulting in opening of voltage gated Ca^{2+} channels (VDCC) (McClenaghan, 2007, Drews *et al.* 2010, Fu *et al.* 2013). Ca^{2+} oscillations stimulate pulsatile insulin secretion with exocytosis of secretory granules which accounts for the first and early phase of insulin secretion. K_{ATP} channel independent mechanisms (Ca^{2+} dependent or independent) mediate the second phase of insulin secretion. The K_{ATP} channel dependent pathway is considered to be the major trigger for glucose stimulated insulin secretion (GSIS), with amplification by pathways triggered by adenylate cyclase (cAMP, PKA) or phospholipase C (PKC) (Yaney *et al.* 2002, Doyle & Egan, 2007).

Inhibitors of enzymes (AC, PLC) and ion channels (K_{ATP}, VDCC), fluorescent dyes to monitor membrane potential and intracellular Ca^{2+}, measurement of second messengers such as cyclic AMP and electrophysiological techniques are useful to delineate mechanisms underlying the insulinotropic actions of novel peptides and drugs (Yaney *et al.* 2002, Miguel *et al.* 2004, Drews *et al.* 2010, Hodson *et al.* 2014). We used these strategies to understand better the actions through which esculentin-2CHa(1-30) and its selected analogues elicited insulin secretion using BRIN-BD11 cells. Direct measurement of cyclic AMP showed that unlike GLP-1 (Dyachok *et al.* 2006, Ramos *et al.* 2008), esculentin-2CHa(1-30) peptides had little effect on cyclic AMP, resembling the actions of CCK-8. Consistent with this, downregulation of PKC pathway after overnight culture with PMA (Yaney *et al.* 2002) significantly reduced PMA, GLP-1, pggCCK8, Peptide 1, Peptide 6 and Peptide 9 induced insulin secretion. Similarly AC inhibition using NKY80 reduced GLP-1 induced insulin release but not the stimulatory effects of pggCCK8 or esculentin-2CHa(1-30) peptides.

To establish involvement of ionic events, we studied the actions of diazoxide, high K^+ solution, verapamil and depletion of Ca^{2+} on the effects of esculentin-2CHa(1-30) peptides. Each of these conditions inhibited the insulinotropic response. Consistent with these data, the insulin-secretory effects of the peptides on BRIN-BD11 cells were accompanied by depolarisation and increased intracellular Ca^{2+}.–Collectively, these findings suggested to us that the insulinotropic effects of esculentin-2CHa(1-30) peptides might result, at least in part, from the inhibition of KATP channels to cause depolarisation and voltage-dependent Ca^{2+} influx. In patch-clamp experiments, however, we found that esculentin-2CHa(1-30) peptides had no direct effect on beta cell KATP channels. This raises the possibility of an action on other ion channels such as L-type Ca^{2+} channels a direct depolarising effect resulting from positively charged peptides entering the beta cell as suggested by imaging studies using fluorescently tagged FITC-esculentin-2CHa(1-30). Further studies will be required to evaluate such effects and the consequences of longer term exposure of beta cells to these peptides.

Cell-penetrating peptides are receiving increasing interest as vehicles for intracellular delivery of therapeutic agents such as anti-cancer drugs (Kurrikoff et al . 2016). The relatively rapid and efficient internalization of FITC-esculentin-2CHa(1-30) by BRIN-BD11 cells, without loss of integrity of the plasma membrane, suggests a possible application for enzyme-resistant analogues of the peptide. In this regard, esculentin-2CHa(1-30) resembles the amphibian histone H2A-derived peptide buforin II (Elmore. 2012). Buforin II traverses the cell membrane in a cooperative manner without producing significant damage by a mechanism that involves formation of transient toroidal pore structures. Once internalized, buforin II accumulates in the nucleus and alters cellular function (Lee *et al*. 2008). Studies *in vivo* (unpublished data) have shown that treatment of high fat-fed mice with esculentin-2CHa(1-30) and its analogues ameliorates diabetes and has beneficial effects on expression of pancreatic islet genes involved with insulin release suggesting that the internalized peptide may also be able to regulate transcription.

In conclusion, the present study has shown that analogues of esculentin-2CHa(1-30), namely [D-Arg^7, D-Lys^{15}, D-Lys^{23}]-esculentin-2CHa(1-30) and Lys^{15}-octanoate-esculentin-2CHa(1-30) (Peptides 6 and 9 respectively demonstrate enhanced resistance to degradation by endopeptidases and strong insulinotropic actions on rat and human clonal beta cells as well as primary mouse islets. These peptide analogues also exerted anti-hyperglycaemic effects and promoted glucose-induced insulin release normal mice. Detailed studies investigating the effects of chronic administration of these peptides in animal models of obesity-diabetes are needed to further explore the potential of esculentin-2CHa(1-30) analogues for therapy of diabetes in man.

Author Contributions

SV, MKM, RCM performed experiments, analysed data and prepared the manuscript. TMC, JMC, YHAA and PRF conceived and designed the study and prepared the manuscript.

Acknowledgements

Funding for this study was provided by a proof of concept project grant from Invest NI (Grant Number POC 418) and project grant from Diabetes UK.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Abdel-Wahab YH, Flatt PR, Patterson S & Conlon JM 2010 Insulin-releasing properties of the frog skin peptide B2RP (brevinin-2 related peptide) and its analogues both in vitro and in vivo. *Regul Pept* **164** 51.

Abdel-Wahab YH, Power GJ, Ng MT, Flatt PR & Conlon JM 2008 Insulin-releasing properties of the frog skin peptide pseudin-2 and its [Lys18]-substituted analogue.*Biol Chem* **389** 143-148.

Attoub S, Mechkarska M, Sonnevend A, Radosavljevic G, Jovanovic I, Lukic ML & Conlon JM 2013 Esculentin-2CHa: a host-defense peptide with differential cytotoxicity against bacteria, erythrocytes and tumor cells. *Peptides* **39** 95-102.

Bailey CJ 2009 New therapies for diabesity. *Curr Diab Rep* **9** 360-367.

Conlon JM 2008 Reflections on a systematic nomenclature for antimicrobial peptides from the skins of frogs of the family Ranidae. *Peptides* **29** 1815-1819.

Conlon JM 2011 Structural diversity and species distribution of host-defense peptides in frog skin secretions. *Cell Mol Life Sci* **68** 2303-2315.

Conlon JM & Mechkarska M 2014 Host-defense peptides with therapeutic potential from skin secretions of frogs from the family pipidae. *Pharmaceuticals (Basel)* **15** 58-77.

Conlon JM, Mechkarska M, Coquet L, Jouenne T, Leprince J, Vaudry H, Kolodziejek J, Nowotny N & King JD 2011 Characterization of antimicrobial peptides in skin secretions from discrete populations of Lithobates chiricahuensis (Ranidae) from central and southern Arizona. In *Peptides*, pp 664-669. United States: 2011 Elsevier Inc.

Conlon JM, Mechkarska M, Lukic ML & Flatt PR 2014 Potential therapeutic applications of multifunctional host-defense peptides from frog skin as anti-cancer, anti-viral, immunomodulatory, and anti-diabetic agents. *Peptides* **57** 67-77.

Conlon JM, Patterson S & Flatt PR 2006 Major contributions of comparative endocrinology to the development and exploitation of the incretin concept. *J Exp Zool A Comp Exp Biol* **305** 781-786.

Doyle ME & Egan JM 2007 Mechanisms of action of glucagon-like peptide 1 in the pancreas. *Pharmacol Ther* **113** 546-593.

Drews G, Krippeit-Drews P & Dufer M 2010 Electrophysiology of islet cells. *Adv Exp Med Biol* **654** 115-163.

Dyachok O, Isakov Y, Sagetorp J & Tengholm A 2006 Oscillations of cyclic AMP in hormone-stimulated insulin-secreting beta-cells. *Nature* **439** 349-352.

Elmore DE 2012 Insights into buforin II membrane translocation from molecular dynamics simulations. Peptides. **38** 357-362

Flatt PR & Bailey CJ 1981a Abnormal plasma glucose and insulin responses in heterozygous lean (ob/+) mice. *Diabetologia* **20** 573-577.

Flatt PR & Bailey CJ 1981b Development of glucose intolerance and impaired plasma insulin response to glucose in obese hyperglycaemic (ob/ob) mice. *Horm Metab Res* **13** 556-560.

Fu A, Eberhard CE & Screaton RA 2013 Role of AMPK in pancreatic beta cell function. *Mol Cell Endocrinol* **366** 127-134.

Gotoh M, Maki T, Kiyoizumi T, Satomi S & Monaco AP 1985 An improved method for isolation of mouse pancreatic islets. *Transplantation* **40** 437-438.

Green AD, Vasu S, McClenaghan NH & Flatt PR 2015 Pseudoislet formation enhances gene expression, insulin secretion and cytoprotective mechanisms of clonal human insulin-secreting 1.1B4 cells. *Pflugers Arch* **467** 2219-2228.

Hodson DJ, Tarasov AI, Gimeno Brias S, Mitchell RK, Johnston NR, Haghollahi S, Cane MC, Bugliani M, Marchetti P, Bosco D, et al. 2014 Incretin-modulated beta cell energetics in intact islets of Langerhans. *Mol Endocrinol* **28** 860-871.

Irwin N & Flatt PR 2015 New perspectives on exploitation of incretin peptides for the treatment of diabetes and related disorders. *World J Diabetes* **6** 1285-1295.

Irwin N, Frizelle P, O'Harte FP & Flatt PR 2013 (pGlu-Gln)-CCK-8[mPEG]: a novel, long-acting, mini-PEGylated cholecystokinin (CCK) agonist that improves metabolic status in dietary-induced diabetes. *Biochim Biophys Acta* **1830** 4009-4016.

Jackway RJ, Pukala TL, Donnellan SC, Sherman PJ, Tyler MJ & Bowie JH 2011 Skin peptide and cDNA profiling of Australian anurans: genus and species identification and evolutionary trends. *Peptides* **32** 161-172.

Kahn SE, Cooper ME & Del Prato S 2014 Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future. *Lancet* **383** 1068-1083.

Kurrikoff K, Gestin M, Langel Ü. 2016 Recent in vivo advances in cell-penetrating peptide-assisted drug delivery. Expert Opin Drug Deliv. 13 :373-387.

Lacy PE & Kostianovsky M 1967 Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* **16** 35-39.

Lee HS, Park CB, Kim JM, Jang SA, Park IY, Kim MS, Cho JH, Kim SC (2008) Mechanism of anticancer activity of buforin IIb, a histone H2A-derived peptide. Cancer Lett 271 47-55.

McCarthy MI 2011 Dorothy Hodgkin Lecture 2010. From hype to hope? A journey through the genetics of Type 2 diabetes. *Diabet Med* **28** 132-140.

McClenaghan NH 2007 Physiological regulation of the pancreatic {beta}-cell: functional insights for understanding and therapy of diabetes. In *Exp Physiol*, pp 481-496. England.

McClenaghan NH, Barnett CR, Ah-Sing E, Abdel-Wahab YH, O'Harte FP, Yoon TW, Swanston-Flatt SK & Flatt PR 1996 Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-BD11, produced by electrofusion. *Diabetes* **45** 1132-1140.

McClenaghan NH, Flatt PR & Ball AJ 2006 Actions of glucagon-like peptide-1 on KATP channel-dependent and -independent effects of glucose, sulphonylureas and nateglinide. *J Endocrinol* **190** 889-896.

McCluskey JT, Hamid M, Guo-Parke H, McClenaghan NH, Gomis R & Flatt PR 2011 Development and functional characterization of insulin-releasing human pancreatic beta cell lines produced by electrofusion. *J Biol Chem* **286** 21982-21992.

Mechkarska M, Ahmed E, Coquet L, Leprince J, Jouenne T, Vaudry H, King JD & Conlon JM 2010 Antimicrobial peptides with therapeutic potential from skin secretions of the Marsabit clawed frog *Xenopus borealis* (Pipidae). *Comp Biochem Physiol C Toxicol Pharmacol* **152** 467-472.

Mechkarska M, Ojo OO, Meetani MA, Coquet L, Jouenne T, Abdel-Wahab YH, Flatt PR, King JD & Conlon JM 2011 Peptidomic analysis of skin secretions from the *Lithobates catesbeianus* (Ranidae) identifies multiple peptides with potent insulin-releasing activity. *Peptides* **32** 203-208.

Miguel JC, Patterson S, Abdel-Wahab YH, Mathias PC & Flatt PR 2004 Time-correlation between membrane depolarization and intracellular calcium in insulin secreting BRIN-BD11 cells: studies using FLIPR. *Cell Calcium* **36** 43-50.

Nicolas P & El Amri C 2009 The dermaseptin superfamily: a gene-based combinatorial library of antimicrobial peptides. In *Biochim Biophys Acta*, pp 1537-1550. Netherlands.

O'Harte FP, Mooney MH, Kelly CM, McKillop AM & Flatt PR 2001 Degradation and glycemic effects of His(7)-glucitol glucagon-like peptide-1(7-36)amide in obese diabetic ob/ob mice. *Regul Pept* **96** 95-104.

Ojo OO, Abdel-Wahab YH, Flatt PR & Conlon JM 2013 Insulinotropic actions of the frog skin host-defense peptide alyteserin-2a: a structure-activity study. *Chem Biol Drug Des* **82** 196-204.

Ojo OO, Abdel-Wahab YH, Flatt PR, Mechkarska M & Conlon JM 2011 Tigerinin-1R: a potent, non-toxic insulin-releasing peptide isolated from the skin of the Asian frog, Hoplobatrachus rugulosus. *Diabetes Obes Metab* **13** 1114-1122.

Ojo OO, Srinivasan DK, Owolabi BO, Conlon JM, Flatt PR & Abdel-Wahab YH 2015a Magainin-AM2 improves glucose homeostasis and beta cell function in high-fat fed mice. *Biochim Biophys Acta* **1850** 80-87.

Ojo OO, Srinivasan DK, Owolabi BO, Flatt PR & Abdel-Wahab YH 2015b Beneficial effects of tigerinin-1R on glucose homeostasis and beta cell function in mice with diet-induced obesity-diabetes. *Biochimie* **109** 18-26.

Ojo OO, Srinivasan DK, Owolabi BO, McGahon MK, Moffett RC, Curtis TM, Conlon JM, Flatt PR & Abdel-Wahab YH 2016 Molecular mechanisms mediating the beneficial metabolic effects of [Arg4]tigerinin-1R in mice with diet-induced obesity and insulin resistance. *Biol Chem* **397** 753-764.

Ojo OO, Srinivasan DK, Owolabi BO, Vasu S, Conlon JM, Flatt PR & Abdel-Wahab YH 2015c Esculentin-2CHa-Related Peptides Modulate Islet Cell Function and Improve Glucose Tolerance in Mice with Diet-Induced Obesity and Insulin Resistance. *PLoS One* **10** e0141549.

Owolabi BO, Ojo OO, Srinivasan DK, Conlon JM, Flatt PR & Abdel-Wahab YH 2016 In vitro and in vivo insulinotropic properties of the multifunctional frog skin peptide hymenochirin-1B: a structure-activity study. *Amino Acids* **48** 535-547.

Parkes DG, Mace KF & Trautmann ME 2013 Discovery and development of exenatide: the first antidiabetic agent to leverage the multiple benefits of the incretin hormone, GLP-1. *Expert Opin Drug Discov* **8** 219-244.

Ramos LS, Zippin JH, Kamenetsky M, Buck J & Levin LR 2008 Glucose and GLP-1 stimulate cAMP production via distinct adenylyl cyclases in INS-1E insulinoma cells. *J Gen Physiol* **132** 329-338.

Scholfield CN & Curtis TM 2000 Heterogeneity in cytosolic calcium regulation among different microvascular smooth muscle cells of the rat retina. *Microvasc Res* **59** 233-242.

Srinivasan D, Ojo OO, Owolabi BO, Conlon JM, Flatt PR & Abdel-Wahab YH 2015 The frog skin host-defense peptide CPF-SE1 improves glucose tolerance, insulin sensitivity and islet function and decreases plasma lipids in high-fat fed mice. *Eur J Pharmacol* **764** 38-47.

Stumvoll M, Goldstein BJ & van Haeften TW 2008 Type 2 diabetes: pathogenesis and treatment. *Lancet* **371** 2153-2156.

Yaney GC, Fairbanks JM, Deeney JT, Korchak HM, Tornheim K & Corkey BE 2002 Potentiation of insulin secretion by phorbol esters is mediated by PKC-alpha and nPKC isoforms. *Am J Physiol Endocrinol Metab* **283** E880-888.

Table 1 Amino acid sequences and molecular masses of esculentin-2CHa, esculentin-2CHa(1-30) and substituted analogues

Peptide No.	**Name**	**Primary Sequence**	**Theoretical molecular mass (Da)**	**Measured molecular mass (Da)**
	Esculentin-2CHa	GFSSIFRGVAKFASKGLGKDLAKLGVDLVACKISKQC	3841.6	-
1	Esculentin-2CHa-(1-30)	GFSSIFRGVAKFASKGLGKDLAKLGVDLVA	3052.6	3053.7
2	[D-Arg^{7}]-Esculentin-2CHa-(1-30)	GFSSIF**R**GVAKFASKGLGKDLAKLGVDLVA	3052.6	3053.1
3	[D-Lys^{15}]-Esculentin-2CHa-(1-30)	GFSSIFRGVAKFAS**K**GLGKDLAKLGVDLVA	3052.6	3052.0
4	[D-Lys^{23}]-Esculentin-2CHa-(1-30)	GFSSIFRGVAKFASKGLGKDLA**K**LGVDLVA	3052.6	3054.0
5	[D-Lys^{15},D-Lys^{23}]-Esculentin-2CHa-(1-30)	GFSSIFRGVAKFAS**K**GLGKDLA**K**LGVDLVA	3052.6	3053.8
6	[D-Arg^{7}, D-Lys^{15},D-Lys^{23}]-Esculentin-2CHa-(1-30)	GFSSIF**R**GVAKFAS**K**GLGKDLA**K**LGVDLVA	3052.6	3053.9
7	[L-Orn^{15}, L-Orn^{23}]-Esculentin-2CHa-(1-30)	GFSSIFRGVAKFAS**Orn**GLGKDLA**Orn**LGVDLVA	3024.5	3026.3
8	Esculentin-2CHa-(1-30)-NH_2	GFSSIFRGVAKFASKGLGKDLAKLGVDLVA-**NH_2**	3051.6	3051.0
9	Lys^{15}-octanoate -Esculentin-2CHa-(1-30)	GFSSIFRGVAKFAS**K(Oct)**GLGKDLAKLGVDLVA	3178.6	3177.5
10	Lys^{23}-octanoate -Esculentin-2CHa-(1-30)	GFSSIFRGVAKFASKGLGKDLAK**(Oct)**LGVDLVA	3178.6	3176.6

Table 2 Degradation of esculentin-2CHa(1-30) peptides in plasma and effects on insulin and LDH release from clonal BRIN BD11 cells compared with established secretagogues

Secretagogue/Peptide	% Degradation (8 h in mouse plasma)	BRIN-BD11 cells		
		Insulin release (ng/million cells/20 min)	Threshold concentration	LDH release (% of control)
Glucose (5.6 mM)	--	0.75 ± 0.04	--	102.8 ± 5.4
Glucose (16.7 mM)	--	1.36 ± 0.10***	--	106.9 ± 1.3
Alanine (10 mM)	--	5.00 ± 0.50***	--	106.1 ± 1.8
GLP-1 (7-36) NH$_2$ (10^{-6} M)	--	1.96 ± 0.17***	--	94.7 ± 5.3
Peptide 1 (3 x 10^{-6} M)	93	1.32 ± 0.04***	10^{-7} M	105.9 ± 5.7
Peptide 2 (3 x 10^{-6} M)	59	1.57 ± 0.04$^{***, \Delta\Delta}$	3 x 10^{-7} M	128.2 ± 5.4***
Peptide 3 (3 x 10^{-6} M)	46	1.06 ± 0.08$^{**, \Delta\Delta}$	3 x 10^{-6} M	122.6 ± 1.4
Peptide 4 (3 x 10^{-6} M)	80	1.22 ± 0.03$^{***, \Delta\Delta}$	3 x 10^{-7} M	107.6 ± 4.6
Peptide 5 (3 x 10^{-6} M)	81	1.06 ± 0.04$^{**, \Delta\Delta}$	10^{-6} M	90.1 ± 1.6
Peptide 6 (3 x 10^{-6} M)	24	1.96 ± 0.08$^{***, \Delta\Delta}$	10^{-6} M	114.6 ± 5.9
Peptide 7 (3 x 10^{-6} M)	94	2.75 ± 0.09$^{***, \Delta\Delta\Delta}$	3 x 10^{-7} M	100.1 ± 4.2
Peptide 8 (3 x 10^{-6} M)	92	1.13 ± 0.09$^{*,\Delta}$	3 x 10^{-6} M	92.9 ± 8.8
Peptide 9 (3 x 10^{-6} M)	62	2.47 ± 0.12$^{***, \Delta\Delta}$	3 x 10^{-6} M	105.0 ± 6.8
Peptide 10 (3 x 10^{-6} M)	79	1.65 ± 0.15***	10^{-6} M	106.8 ± 4.3

Values are mean ± SEM (n=8). *p<0.05, **p<0.01, ***p<0.001 compared to respective control at glucose (5.6 mM). $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01, $^{\Delta\Delta\Delta}$p<0.001 compared to esculentin-2CHa(1-30) (Peptide 1).

Figure 1

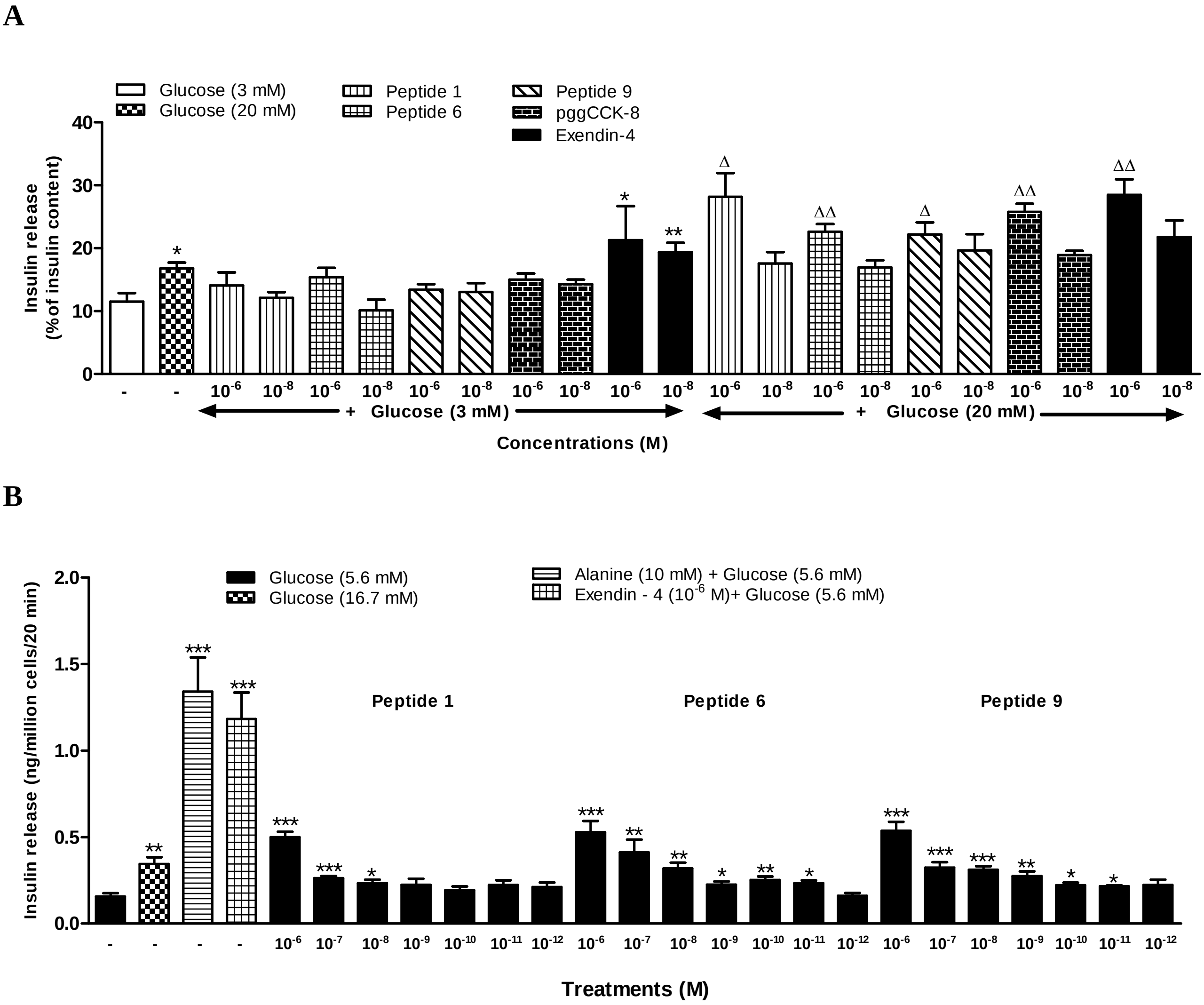

A
Glucose (3 mM)
Glucose (20 mM)
Peptide 1
Peptide 6
Peptide 9
pggCCK-8
Exendin-4
Insulin release
(% of insulin content)
40
30
20
10
0
*
*
**
Δ
ΔΔ
Δ
ΔΔ
ΔΔ
-
-
10-6
10-8
+ Glucose (3 mM)
+ Glucose (20 mM)
Concentrations (M)
B
Glucose (5.6 mM)
Glucose (16.7 mM)
Alanine (10 mM) + Glucose (5.6 mM)
Exendin - 4 (10-6 M)+ Glucose (5.6 mM)
Insulin release (ng/million cells/20 min)
2.0
1.5
1.0
0.5
0.0
Peptide 1
Peptide 6
Peptide 9
**
***
***
***
***
*
***
**
**
*
**
*
***
***
***
**
*
*
-
10-6
10-7
10-8
10-9
10-10
10-11
10-12
Treatments (M)

Figure 2

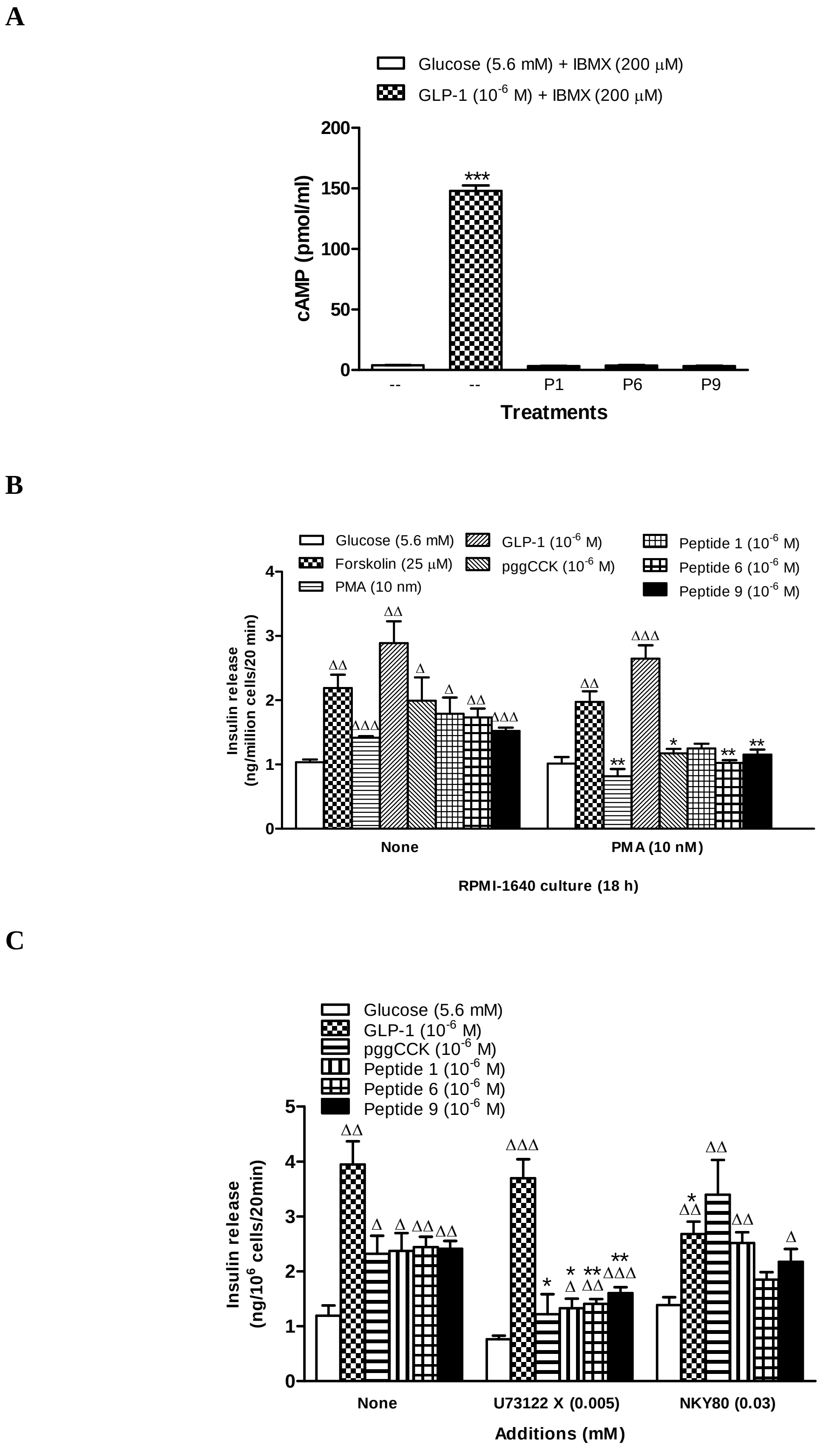
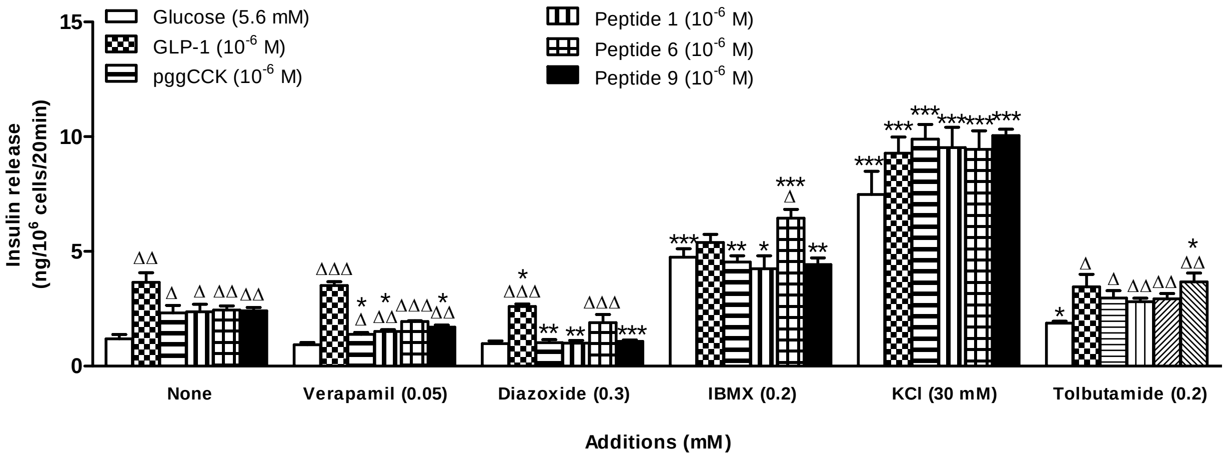

A
Glucose (5.6 mM) + IBMX (200 μM)
GLP-1 (10^{-6} M) + IBMX (200 μM)
cAMP (pmol/ml)
200
150
100
50
0
***
--
--
P1
P6
P9
Treatments
B
Glucose (5.6 mM)
Forskolin (25 μM)
PMA (10 nm)
GLP-1 (10^{-6} M)
pggCCK (10^{-6} M)
Peptide 1 (10^{-6} M)
Peptide 6 (10^{-6} M)
Peptide 9 (10^{-6} M)
Insulin release
(ng/million cells/20 min)
4
3
2
1
0
ΔΔ
ΔΔΔ
ΔΔ
Δ
Δ
ΔΔ
ΔΔΔ
ΔΔ
**
ΔΔΔ
*
**
**
None
PMA (10 nM)
RPMI-1640 culture (18 h)
C
Glucose (5.6 mM)
GLP-1 (10^{-6} M)
pggCCK (10^{-6} M)
Peptide 1 (10^{-6} M)
Peptide 6 (10^{-6} M)
Peptide 9 (10^{-6} M)
Insulin release
(ng/10^{6} cells/20min)
5
4
3
2
1
0
ΔΔ
Δ
Δ
ΔΔ
ΔΔ
ΔΔΔ
*
*
Δ
**
ΔΔ
**
ΔΔΔ
*
ΔΔ
ΔΔ
ΔΔ
Δ
None
U73122 X (0.005)
NKY80 (0.03)
Additions (mM)

Figure 3

A


Glucose (5.6 mM)
GLP-1 (10^{-6} M)
pggCCK (10^{-6} M)
Peptide 1 (10^{-6} M)
Peptide 6 (10^{-6} M)
Peptide 9 (10^{-6} M)
Insulin release (ng/10^{6} cells/20min)
15
10
5
0
None
Verapamil (0.05)
Diazoxide (0.3)
IBMX (0.2)
KCl (30 mM)
Tolbutamide (0.2)
Additions (mM)
ΔΔ Δ Δ ΔΔ ΔΔ
ΔΔΔ * Δ * ΔΔ ΔΔΔ * ΔΔ
* ΔΔΔ ** ** ΔΔΔ ***
*** ** * *** Δ **
*** *** *** *** *** ***
* Δ Δ ΔΔ ΔΔ * ΔΔ

B

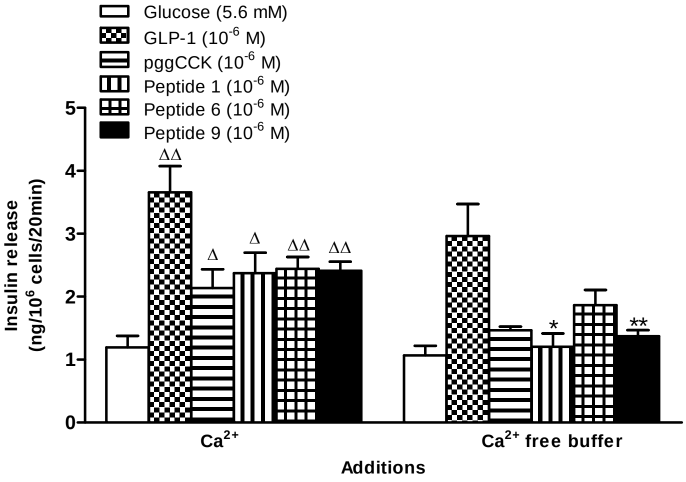

Glucose (5.6 mM)
GLP-1 (10^{-6} M)
pggCCK (10^{-6} M)
Peptide 1 (10^{-6} M)
Peptide 6 (10^{-6} M)
Peptide 9 (10^{-6} M)
Insulin release (ng/10^{6} cells/20min)
5
4
3
2
1
0
ΔΔ
Δ
Δ
ΔΔ
ΔΔ
*
**
Ca^{2+}
Ca^{2+} free buffer
Additions

Figure 4

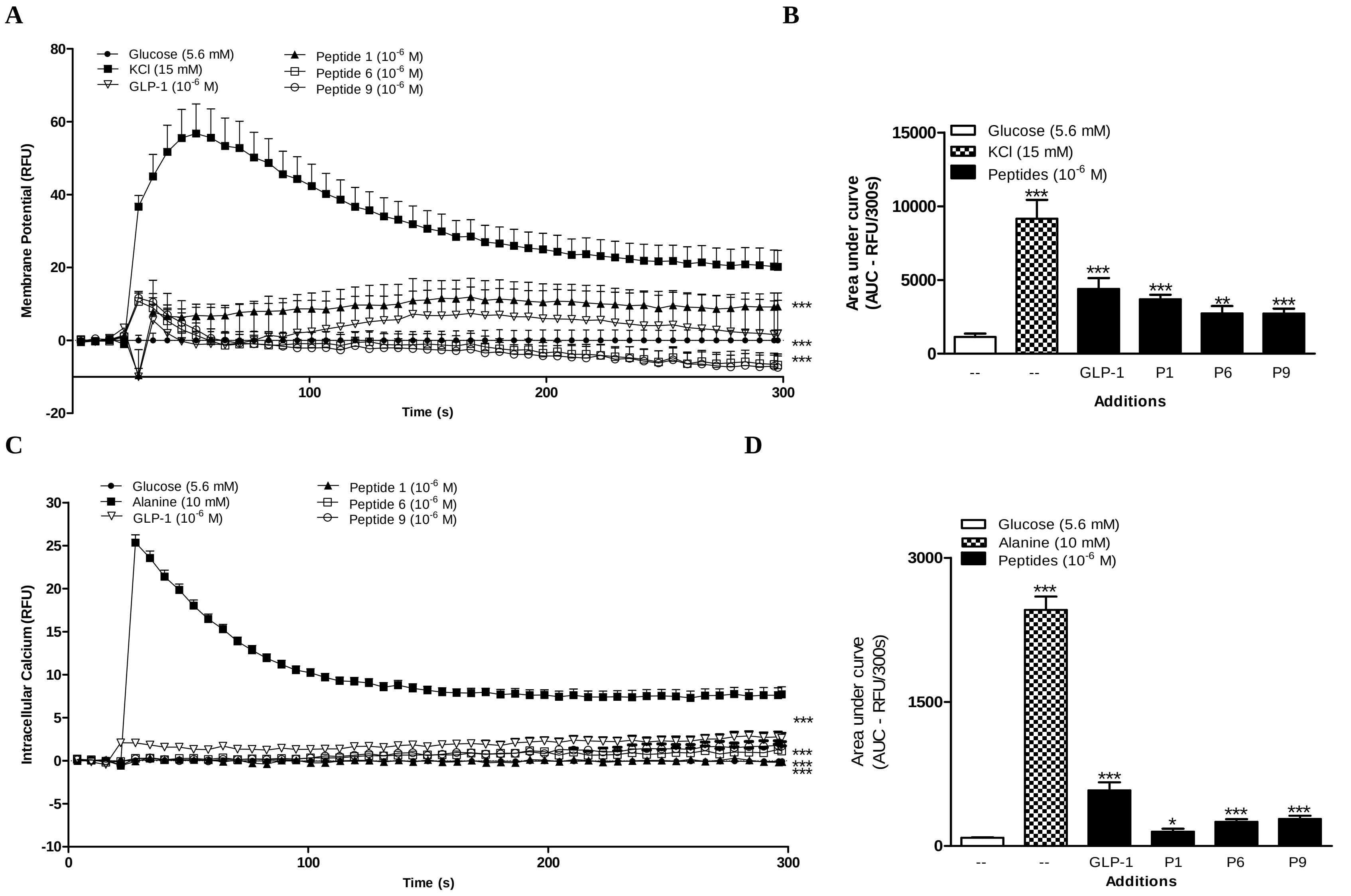

A
Membrane Potential (RFU)
Time (s)
Glucose (5.6 mM)
KCl (15 mM)
GLP-1 (10^{-6} M)
Peptide 1 (10^{-6} M)
Peptide 6 (10^{-6} M)
Peptide 9 (10^{-6} M)
80
60
40
20
0
-20
100
200
300
***
***
***
B
Area under curve (AUC - RFU/300s)
Glucose (5.6 mM)
KCl (15 mM)
Peptides (10^{-6} M)
15000
10000
5000
0
--
--
GLP-1
P1
P6
P9
Additions
***
***
***
**
***
C
Intracellular Calcium (RFU)
Time (s)
Glucose (5.6 mM)
Alanine (10 mM)
GLP-1 (10^{-6} M)
Peptide 1 (10^{-6} M)
Peptide 6 (10^{-6} M)
Peptide 9 (10^{-6} M)
30
25
20
15
10
5
0
-5
-10
0
100
200
300
***
***
***
***
D
Area under curve (AUC - RFU/300s)
Glucose (5.6 mM)
Alanine (10 mM)
Peptides (10^{-6} M)
3000
1500
0
--
--
GLP-1
P1
P6
P9
Additions
***
***
*
***
***

Figure 5

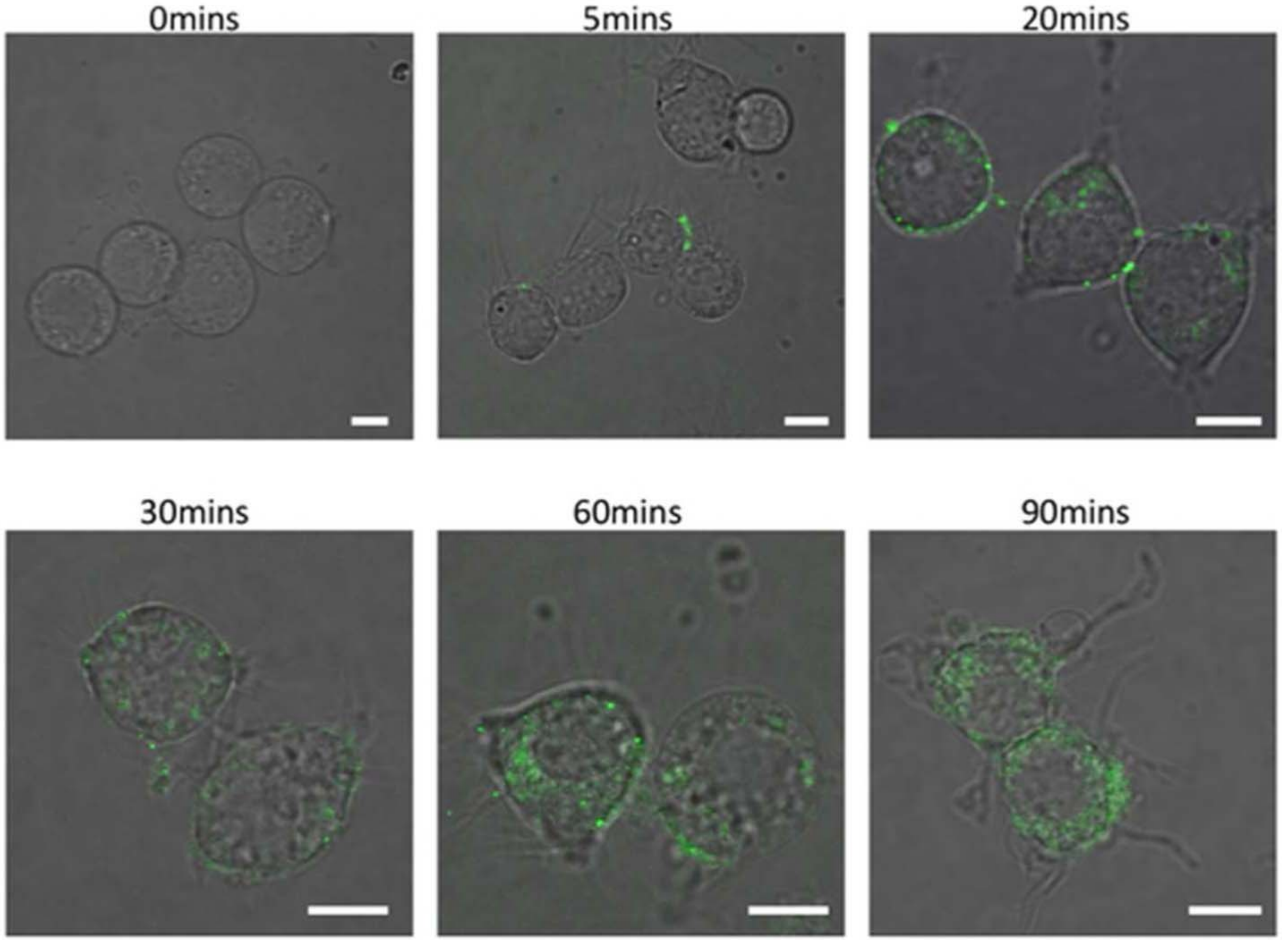


Figure 6

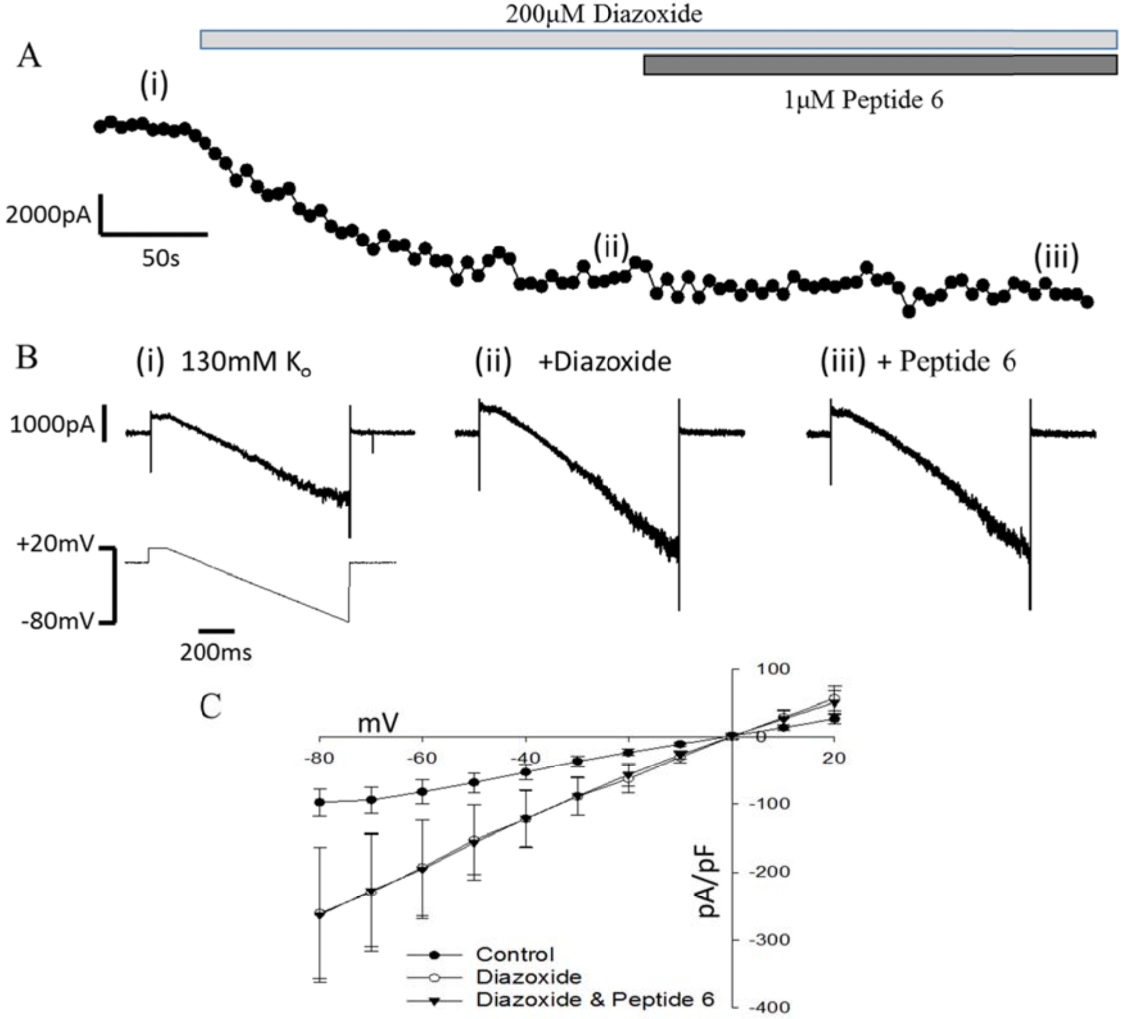
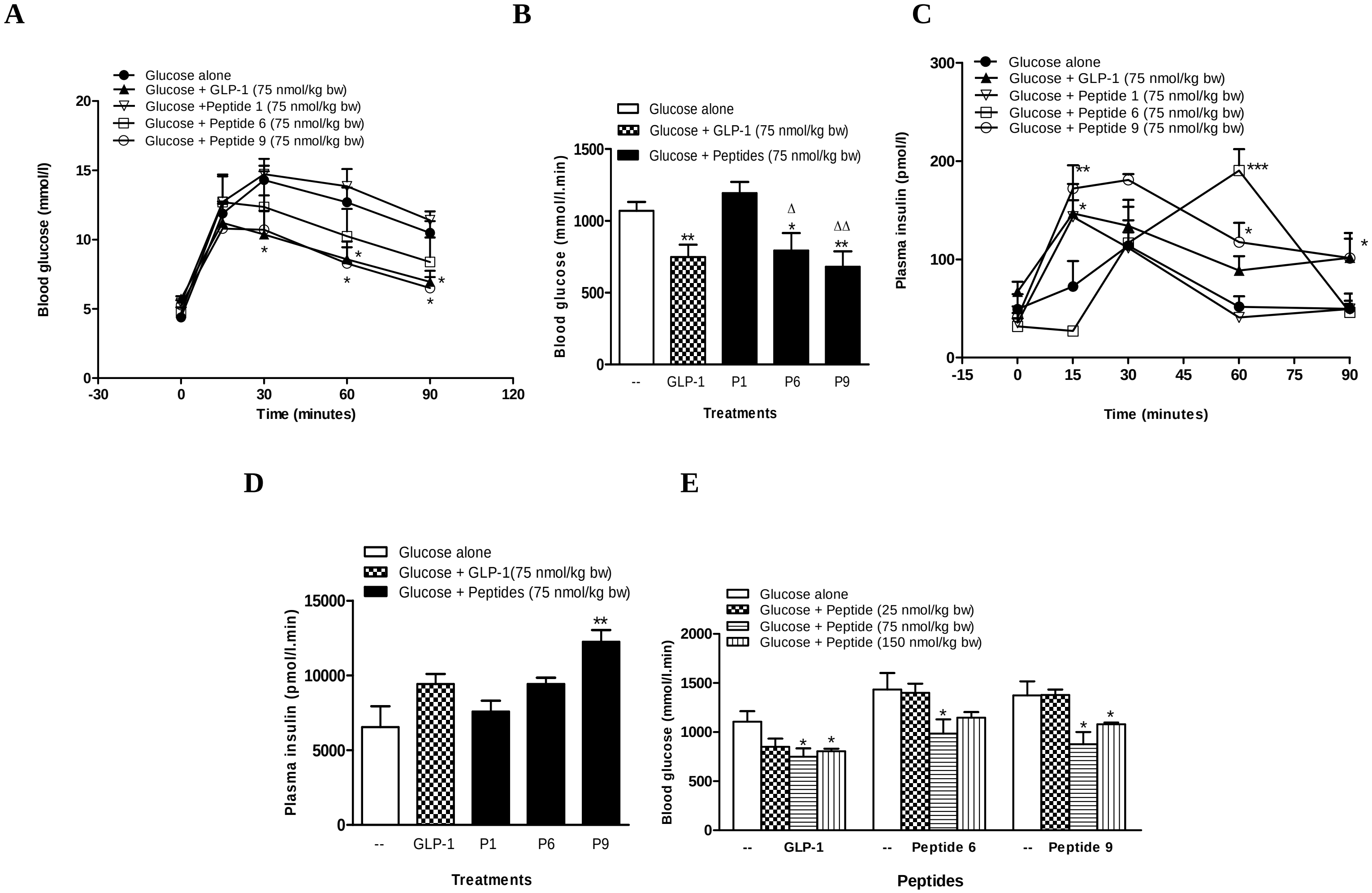

200μM Diazoxide
A
(i)
1μM Peptide 6
2000pA
50s
(ii)
(iii)
B
(i) 130mM K$_o$
(ii) +Diazoxide
(iii) + Peptide 6
1000pA
+20mV
-80mV
200ms
C
mV
-80
-60
-40
0
20
100
-100
-200
-300
-400
pA/pF
Control
Diazoxide
Diazoxide & Peptide 6

Figure 7


A
Glucose alone
Glucose + GLP-1 (75 nmol/kg bw)
Glucose +Peptide 1 (75 nmol/kg bw)
Glucose + Peptide 6 (75 nmol/kg bw)
Glucose + Peptide 9 (75 nmol/kg bw)
Blood glucose (mmol/l)
Time (minutes)
0
5
10
15
20
-30
0
30
60
90
120
*
B
Glucose alone
Glucose + GLP-1 (75 nmol/kg bw)
Glucose + Peptides (75 nmol/kg bw)
Blood glucose (mmol/l.min)
0
500
1000
1500
--
GLP-1
P1
P6
P9
Treatments
**
Δ
ΔΔ
C
Glucose alone
Glucose + GLP-1 (75 nmol/kg bw)
Glucose + Peptide 1 (75 nmol/kg bw)
Glucose + Peptide 6 (75 nmol/kg bw)
Glucose + Peptide 9 (75 nmol/kg bw)
Plasma insulin (pmol/l)
0
100
200
300
-15
0
15
30
45
60
75
90
Time (minutes)
***
D
Glucose alone
Glucose + GLP-1(75 nmol/kg bw)
Glucose + Peptides (75 nmol/kg bw)
Plasma insulin (pmol/l.min)
0
5000
10000
15000
--
GLP-1
P1
P6
P9
Treatments
E
Glucose alone
Glucose + Peptide (25 nmol/kg bw)
Glucose + Peptide (75 nmol/kg bw)
Glucose + Peptide (150 nmol/kg bw)
Blood glucose (mmol/l.min)
0
500
1000
1500
2000
--
GLP-1
Peptide 6
Peptide 9
Peptides